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14. ABSTRACT: A lysate of an immortalized monoclonal cell line derived from the striatum (X61) contains an activity that is capable of increasing the dopamine content of an immortalized, dopaminergic mouse mesencephalic cell line (MN9D). The activity can be extracted from the cell lysate by isoamyl alcohol/chloroform. NMR spectroscopic analysis demonstrated that a purified fraction from the isoamyl alcohol/chloroform soluble dopaminergic stimulatory activity is a mixture of 80-90% cis-9-octadecenoic acid (oleic acid) and 10-20% cis-11-octadecenoic acid (cis-vaccenic acid). MN9D dopamine increases linearly in the presence of oleic acid over a 48 hour period suggesting the induction of an increased dopaminergic phenotype in these dividing cells. The ability to increase MN9D dopamine by oleic and cis-vaccenic acids is shared by a number of other long-chain fatty acids including arachidonic, linoleic, linolenic, palmitoleic, and cis-13-octadecenoic acid. Studies on the mechanism of elevation of MN9D dopamine by oleic acid suggest that this fatty acid increases both the synthesis and uptake and/or storage of dopamine. The possibility that oleic or other relatively innocuous fatty acids might affect dopaminergic function in primary neurons is intriguing with respect to possible therapeutic approaches to the treatment of dopaminergic cell loss and the motor sequelae of Parkinson's disease.

15. SUBJECT TERMS
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INTRODUCTION

This report covers the research conducted under Award Number DAMD17-01-1-0819 during the period September 15, 2004 to September 14, 2005. Parkinson's disease is a debilitating disorder resulting in severe motor dysfunction including muscle rigidity, bradykinesia and resting tremor. The motor disabilities are a result of degeneration of a dopaminergic projection arising in the mesencephalon and providing an extensive innervation to the corpus striatum a subdivision of brain subserving extrapyramidal motor function. The disease is widespread affecting approximately a million individuals in North America. The prevalence, morbidity and mortality associated with Parkinson's disease has stimulated an intense investigation of a variety of strategies for the treatment of this disorder (for a detailed review see Alexi et al., 2000). Given that it is, as yet, unclear as to what specific molecules will be useful therapeutic agents, investigation of the factors which are involved in the development and maintenance of the nigrostriatal projection remains an active and enlarging field of research and the development of new approaches to the investigation of dopaminergic differentiation, development and cell survival is an essential element in this research endeavor.

Among the treatment modalities currently under investigation is the use of trophic agents capable of increasing dopaminergic cell survival which could act to inhibit or delay the degenerative process. As an approach to the discovery of unique factors capable of increasing dopaminergic function, we have, during the period of Army support under Award Number DAMD17-01-1-0819, succeeded in purifying two types of chemically distinct dopaminergic stimulatory factors from the lysate of the X61 cell, an immortalized hybrid monoclonal cell derived from the corpus striatum. One type of factor is composed of low molecular weight, highly hydrophilic molecules whose specific structures are still under investigation. The second, in contrast, could be extracted by organic solvents from a concentrate of the X61 lysate and was demonstrated to contain primarily the long-chain, unsaturated fatty acid cis-oleic acid. Studies are being conducted on the mechanism of action and potential utility of the small, hydrophilic molecules as well as oleic acid as potential pharmacological approaches to the investigation of the Parkinson's disease and its treatment.

BODY

The primary objective of the research supported by Grant DAMD17-01-1-0819 continues to be the purification, identification and mechanism of action of dopaminergic stimulatory factors obtained from the immortalized monoclonal hybrid cell, X61, derived from the corpus striatum (Heller et al., 2000; Wainwright et al., 1995).

During the period of this report (September 15, 2004 to September 14, 2005), research has been conducted on the following specific tasks outlined in the currently approved Statement of Work. These include: 1) further purification and identification of two, water-soluble dopaminergic stimulatory factors from the lysate of X61 cells as well as an inhibitory factor; 2) examination of the mechanism by which oleic acid and the water-soluble dopaminergic stimulatory factors elevate MN9D dopamine; and 3) examination of the effect of oleic acid on primary monoaminergic neurons maintained in three-dimensional reaggregate culture.

Purification of two, water-soluble, dopaminergic stimulatory activities and one inhibitory activity from X61 cell lysate

As previously described, we have obtained low molecular weight fractions containing dopaminergic stimulatory or inhibitory activity from X61 cell lysate which was allowed to autodigest at room temperature for up to 3 days (see Annual Progress Report of October 2002). The lysate was serially concentrated using a 2.5 L Amicon ultrafiltation device with a YM-5 (5,000 Da cut-off) membrane, followed by dilution with PBS, generating ultrafiltrate fractions (< 5,000 Da) from days zero, 1, 2, and 3. Concentration of the ultrafiltrate fractions from each day of autodigestion was accomplished by Amicon ultrafiltration using a YC-05 (500 Da cut-off) membrane. This procedure generated two fractions: (1) a "YC-05 concentrate" (nominally 500 Da or larger molecules) containing a stimulatory and an inhibitory activity and (2) a "YC-05 ultrafiltrate" (nominally 500 Da or smaller molecules) with a smaller-sized stimulatory activity.

The YC-05 ultrafiltrate fraction from days 2 and 3 of autodigested X61 lysate (representing the majority of the ultrafiltrate stimulatory activity) was further purified by adsorption to charcoal. The charcoal extraction reduced the UV absorbance to about 1% of the initial (at 280 and 260 nm) and to 2% of the initial (at 230 nm), whereas no significant activity was removed by charcoal. The charcoal-adsorbed fraction was then lyophilized and re-dissolved in water at one-fifteenth the initial volume. Multiple, one-ml aliquots were then gel-filtered on a Superdex-peptide column, equilibrated and run in 5 mM sodium phosphate, pH 7.4, 10 mM NaCl, at 0.5 ml/min, and 1.0 ml fractions were collected. The stimulatory activity eluted in fractions 18-20 with the peak in fraction 19. The Superdex-peptide fractions 18-20 were re-extracted with charcoal which further reduced the UV absorbance at 280 and 260 nm to about one-half the initial, and that at 230 nm to about two-thirds the initial, again without significant loss of activity onto charcoal.

The charcoal-absorbed, Superdex-peptide YC-05 ultrafiltrate stimulatory activity was then lyophilized, redissolved in about one-third the initial volume of 0.05% trifluoroacetic acid in water (0.05% TFA) and multiple, 0.50 ml aliquots were chromatographed on a C18 reverse phase column, equilibrated and eluted in 0.05% TFA at 0.2 ml/min. The activity associated transiently with the resin and eluted later and was separate from most of the A215-absorbing material and residual salt. Following purification on the C18 column, the activity was chromatographed in similar fashion (100 ul injected per run) on a C1 (trimethyl, SAS hypersil) column, resulting in considerable additional purification, based upon a reduction of A215 UV-absorbing material.

NMR analysis of this highly purified, YC-05 ultrafiltrate product by Josh Kurutz of the Biomolecular NMR Facility at The University of Chicago has confirmed a high degree of purity and indicated it is a small molecule with a relatively simple structure. Additional studies, underway to determine the precise structure, include mass spectrometry (specifically, determining what sized species correlates best with activity), pKa titration (is there a carboxy or free amine and, if the former, what type of carboxylic acid it might be), and NMR analysis of the purified sample in deuterated dimethylsulfoxide or/and methanol (to avoid spectral signals due to deuterated water).

Dopaminergic stimulatory and inhibitory activities in the YC-05 concentrate (>500 Da) from days 2 and 3 of autodigested X61 lysate were largely separated from each other by chromatography on the Superdex-peptide column, as described above. The inhibitory activity eluted in fractions 17 and 18, whereas the stimulatory was mostly in fraction 19 with smaller amounts in 18 and 20. The stimulatory activity has been further purified by charcoal extraction as described above (reducing the UV absorbance at 280 and 260 nm to less than 0.5% the initial, without loss of activity), re-concentration using a YC-05 membrane (virtually all the stimulatory activity is in the concentrate, >500 Da), followed by a second charcoal extraction. Following lyophilization, the preparation was extracted with methanol, which removed most of the remaining UV absorbance (between 280 and 225 nm) and almost none of the stimulatory activity. NMR analysis (by Josh Kurutz), of a minority of the total activity purified to this stage (but which had not been re-concentrated through a YC-05 membrane following the first charcoal extraction), has demonstrated a fairly simple mixture containing considerable levels of lysine, moderate levels of aspartic acid and threonine, and a small amount of valine (probably all free amino acids and not in polypeptide form). In addition, at least several other additional molecules were present that may not be amino acids. No aromatic or amide hydrogens were apparent. Since none of the identified free amino acids represents the dopaminergic stimulatory activity, their presence may be obscuring the visualization of the NMR spectra corresponding to the activity. Unlike the YC-05 ultrafiltrate stimulatory activity, the stimulatory activity in the concentrate does not bind to a C18 or C1 reverse phase column, which would aid in removal of the free amino acids. Therefore, in order to reduce (or eliminate) the free amino acids in the sample, an additional concentration step has been included between charcoal extractions (as described above), and a second chromatography on the Superdex-peptide column (after methanol extraction) will be employed, followed, if necessary, by one more re-concentrations using the YC-05 membrane. These procedures should allow for the visualization and identification of NMR spectra corresponding to the dopaminergic stimulatory activity. We believe that the two dopaminergic stimulatory activities, one derived from the YC-05 concentrate (>500 Da) and the other from the YC-05 ultrafiltrate fraction (<500 Da), most likely represent different structural molecules based upon their physical properties. The YC-05 ultrafiltrate activity is soluble in methanol and will bind transiently to a C18 or C1 reverse phase HPLC column whereas the YC-05 concentrate activity does not share any of these characteristics.

We have made significant, though preliminary, progress in the purification of the inhibitory activity from the YC-05 concentrate fraction. Following isolation of this activity in fractions 17 and 18 upon Superdex-peptide gel-filtration, it has been chromatographed on a C18 reverse phase column (using the procedure described above for the stimulatory activity in the YC-05 ultrafiltrate). The inhibitor interacts transiently with the resin, eluting later than, and separate from most of the A215 nm UV-absorbing material. Unlike the dopaminergic stimulatory activity in the YC-05 concentrate, contaminants of the inhibitory activity cannot be removed by charcoal adsorption, because the inhibitor itself binds to and is not eluted from the charcoal by any tested solvent. Since this is the case, we will attempt further purification of the inhibitory activity using C1, C8, C4, and CN reverse phase columns, as well as re-chromatography on the C18 column, and follow the purification scheme that appears most advantageous. The purest product obtained will be subjected to NMR analysis by Josh Kurutz and mass spectroscopy by Giri Reddy of the Protein Peptide Facility at the University of Chicago for possible structure identification.

Neurochemical Mechanisms by Which Factors Derived from X61 Cell Lysate Stimulate Dopamine in the MN9D Cell

As previously reported, oleic acid as well as a partially purified, low molecular weight (<5,000 Da) ultrafiltrate fraction (UF) obtained from X61 cell lysate, markedly elevate the cellular dopamine content of MN9D cells. We earlier proposed that the dopaminergic stimulatory effect of these factors on MN9D cells was due an increase in transmitter synthesis and/or dopamine uptake/storage capacity (see Annual Report of December 2004). To examine whether oleic acid affected tyrosine hydroxylase (TH) activity, the rate-limiting enzyme in dopamine synthesis, MN9D cells were treated for 48 hrs with dimethylsulfoxide (DMSO, vehicle), 31 μM, 62 μM or 124 μM oleic acid in the presence or absence of 100 μM n-hydroxybenzylhydrazine dihydrochloride) (NSD-1015), an inhibitor of DOPA decarboxylase (DDC). TH activity was assessed by measuring the accumulation of DOPA in cells and media following treatment with NSD-1015.

The results show that 48 hr exposure of MN9D cells to oleic acid in the absence of NSD-1015 produced a dose-dependent increase (2.3 to 5.7-fold) in MN9D cellular dopamine content as compared to DMSO vehicle control (Table 1). Despite a marked elevation in cellular dopamine by all three concentrations of oleic acid, TH activity, assessed by DOPA accumulation following inhibition of DDC with NSD-1015 only increased modestly (+33%, $p<0.005$) in the cells after exposure to 31 μM oleic acid, but was not affected by higher concentrations.

Table 1

Effect of Oleic Acid on Tyrosine Hydroxylase Activity in MN9D Cells

	DA Cells (ng/mg Pr)	DOPA Accumulation Cells (ng/mg Pr)	DOPA Accumulation Media (μg/mg Pr/ml)
DMSO vehicle	75.47 ± 2.16	193.17 ± 6.26	35.40 ± 0.73
31 μM Oleic acid	172.61 ± 2.01**	257.47 ± 7.76*	39.53 ± 1.33
62 μM Oleic acid	293.07 ± 11.50**	230.04 ± 11.23	38.53 ± 1.94
124 μM Oleic acid	430.12 ± 10.84**	197.49 ± 3.37	44.05 ± 2.42

The values represent the mean ± SEM, n = 3 cultures.
*Significantly different from DMSO vehicle control, $p<0.005$.
**Significantly different from DMSO vehicle control, $p<0.001$.

A similar experiment was carried out to determine whether the ultrafiltrate fraction (UF) affected TH activity in MN9D cells. The UF material used for the studies described in this report on neurochemical mechanisms underlying the dopaminergic stimulatory effect was composed of the low molecular weight fraction of 2 to 3 day autodigested X61 cell lysate which passed through an Amicon YM-5 (MWCO 5,000 Da) membrane. MN9D cells were treated for 48 hrs with phosphate buffered saline (PBS) or with 12 µl or 18 µl of the UF fraction in the presence or absence of 100 µM NSD-1015. Both concentrations of UF markedly increased MN9D cellular dopamine in the absence of NSD-1015 (Table 2). However, TH activity measured in the cells or media, following inhibition of DDC, was not different from the respective PBS control.

Table 2

Effect of the Partially Purified, Low Molecular Weight Ultrafiltrate Fraction (UF) on Tyrosine Hydroxylase Activity in MN9D Cells

	DA Cells (ng/mg Pr)	DOPA Accumulation Cells (ng/mg Pr)	DOPA Accumulation Media (µg/mg Pr/ml)
12 µl PBS	67.39 ± 1.51	226.33 ± 3.77	41.31 ± 1.35
12 µl UF	129.94 ± 2.04*	225.59 ± 27.99	46.40 ± 13.59
18 µl PBS	76.31 ± 1.50	277.27 ± 4.94	41.51 ± 1.11
18 µl UF	197.92 ± 10.52*	267.78 ± 28.83	52.79 ± 6.69

The values represent the mean ± SEM, n = 3 cultures.
*Significantly different from DMSO vehicle control, $p<0.001$.

Although we did not observe an effect of oleic acid on tyrosine hydroxylase activity, we examined whether oleic acid influenced DOPA decarboxylase (DDC) activity, the enzyme responsible for the conversion of DOPA to dopamine. MN9D cells were treated with DMSO or 124 µM oleic acid for 24 hrs and then collected for determination of cellular dopamine content and DDC activity. The assay of DDC activity in cell-free lysates is based on the enzymatic conversion of L-DOPA to dopamine which is measured by HPLC (Nagatsu et al., 1979).

It is apparent that DDC activity does not increase following exposure to oleic acid despite a 5-fold increase in MN9D dopamine content (Table 3).

Table 3

Effect of Oleic Acid on DOPA Decarboxylase (DDC) Activity in MN9D Cells

	Dopamine ng/mg Pr	DDC Activity Dopamine formed ng/mg Pr/min
DMSO	76.08 ± 2.59	82.37 ± 2.88
124 μM Oleic Acid	382.71 ± 10.42*	66.38 ± 9.27

The values represent the mean ± SEM, n = 6 cultures.
*Significantly different from DMSO vehicle control, $p<0.001$.

The results to date indicate that the increase in MN9D dopamine content by oleic acid or the partially purified, low molecular weight ultrafiltrate fraction (UF) is not due to an effect on dopamine synthesis. These findings are also in agreement with immunoblots which demonstrate that the amount of tyrosine hydroxylase protein does not increase when MN9D cells are exposed to oleic acid or to UF (data not shown). In addition, a recent microarray analysis of gene expression in oleic acid treated MN9D cells shows that there is no effect of the fatty acid on tyrosine hydroxylase or DDC mRNA (data not shown).

Given the lack of effect of oleic acid and UF on dopamine synthetic enzymes, an alternative mechanism for the observed increase in MN9D dopamine could be due to increased neurotransmitter uptake and/or storage. This issue was examined by measuring the amount of neurotransmitter in MN9D cells incubated with exogenous dopamine following depletion of endogenous neurotransmitter stores by alpha-methy-p-tyrosine (AMT), an inhibitor of tyrosine hydroxylase. MN9D cells were treated for 48 hrs with PBS, 18 μl UF, DMSO or 124 μM oleic acid. Some of the cultures were exposed to 1 mM AMT for the last 24 hrs to block further dopamine synthesis. At the end of 48 hrs, the cultures were incubated in 100 μM dopamine for 20 min.

Exposure of PBS, UF, DMSO or oleic acid-treated MN9D cells to 1 mM AMT for 24 hrs resulted in significant reductions in cellular dopamine, as compared to non-AMT treated cells, confirming the inhibition of dopamine synthesis (Table 4). A slight elevation in net accumulation of dopamine was observed in MN9D cells treated with UF as compared to PBS control, but this effect was not significant in the presence or absence of AMT. With oleic acid treatment, however, incubation with exogenous neurotransmitter resulted in a significant 2.2-fold increase in net accumulation of dopamine (69.26 ng/mg) in the presence of AMT as compared to DMSO vehicle control (30.88 ng/mg) suggestive of an increase in MN9D dopamine uptake or storage capacity. In the absence of AMT, no difference in dopamine uptake/storage was observed between oleic acid and DMSO-treated MN9D cells. This finding is not unexpected however,

since MN9D cells exposed to 124 μM oleic acid contain a high concentration of dopamine (433.07 ng/mg) and may not be able to store much more additional neurotransmitter.

Table 4

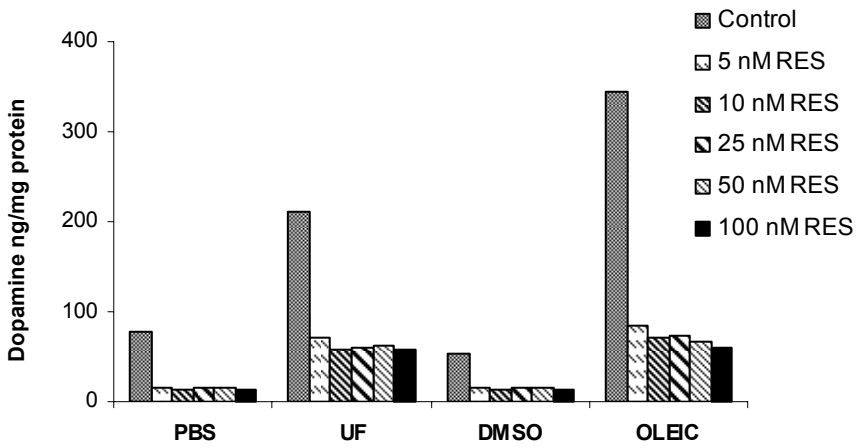
Effect of Ultrafiltrate (UF) or Oleic Acid on Dopamine (DA) Storage Capacity of MN9D Cells

	Endogenous DA ng/mg Pr	**+ AMT** Endogenous DA ng/mg Pr	Net DA Accumulated ng/mg Pr	**+ AMT** Net DA Accumulated ng/mg Pr
PBS	91.25 ± 6.43	3.20 ± 1.00[b]	34.35 ± 4.04	23.20 ± 1.61
UF	168.10 ± 1.16[a]	13.93 ± 0.89[b]	49.05 ± 10.60	31.30 ± 4.26
DMSO	101.56 ± 1.30	1.80 ± 0.24[b]	33.22 ± 4.07	30.88 ± 3.90
Oleic Acid	433.07 ± 10.00[a]	31.86 ± 1.31[b]	23.60 ± 9.39	69.26 ± 5.82*

The values represent the mean ± SEM, n = 3 cultures. Net accumulated dopamine was calculated by subtracting the amount of dopamine in MN9D cells not incubated with exogenous dopamine from corresponding cultures incubated with 100 μM dopamine.
[a]Significantly different from vehicle control, $p<0.001$.
[b]Significantly different from corresponding non-AMT treatment group (i.e., PBS, UF, DMSO, oleic acid), $p<0.001$.
*Significantly different from DMSO vehicle control (+AMT) incubated with dopamine, $p<0.05$.

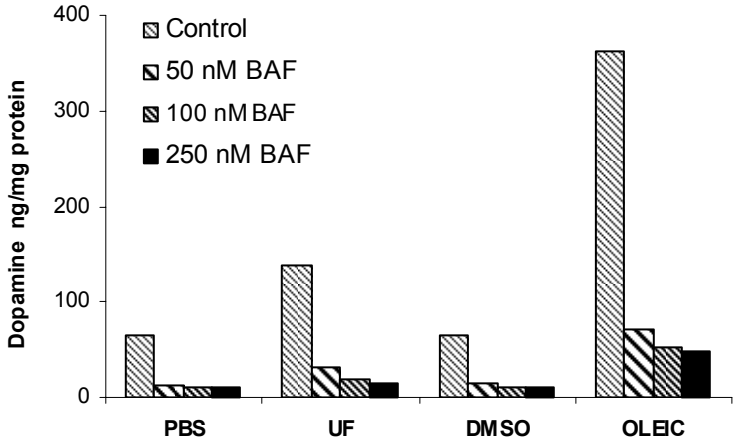
These preliminary data suggest that the elevation in MN9D dopamine seen with oleic acid exposure is due to an increase in dopamine storage capacity. Since catecholamines are normally stored within neurons in secretory vesicles, we examined whether the increase in transmitter within MN9D cells seen with oleic acid or UF treatment was stored within the vesicular compartment. The vesicular transport of catecholamines is mediated by the vesicular monoamine transporter (VMAT2 in mesencephalic dopaminergic neurons) as well as a proton electrochemical gradient generated by a vacuolar H^+-ATPase which is responsible for vesicle acidification and concentration of neurotransmitter. The subcellular location of dopamine storage within MN9D cells was addressed by using reserpine, a VMAT inhibitor, as well as bafilomycin A1 which blocks the vacuolar H^+-ATPase.

Figure 1



MN9D cells were treated with PBS, 25 μL UF, DMSO or 124 μM oleic acid for 48 hrs and then incubated with reserpine (5-100 nM) for 1.5 hrs (Figure 1). Exposure to reserpine markedly reduced MN9D dopamine and the percent reduction in neurotransmitter was roughly similar within each treatment group over a 20-fold range in drug concentration (average reduction: PBS, 80%; UF, 70%; DMSO, 75%; Oleic acid, 80%). Complete depletion of dopamine was observed when MN9D cells were treated with reserpine (5-100 nM) for 48 hrs in combination with PBS, UF, DMSO or oleic acid (data not shown).

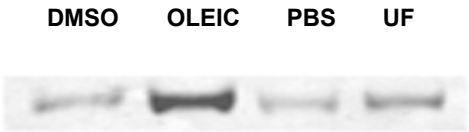
Figure 2



Considerable reductions in dopamine (78-89% of control) were also observed when MN9D cells were treated for 48 hrs with PBS, 18 μL UF, DMSO or 124 μM oleic acid and then incubated with bafilomycin A1 (50-250 nM)) for 1 hr.

The depletion of dopamine by agents which interfere with vesicular storage function by inhibition of VMAT2 (reserpine) or via disruption of the proton electrochemical gradient (bafilomycin A1) demonstrates that the vesicular compartment markedly contributes to neurotransmitter storage in MN9D cells. Apparently, vesicular stores can accommodate a rather sizable increase in cellular dopamine as is the case when MN9D cells are exposed to UF or oleic acid. Evidence that the effect of oleic acid and the UF fraction is mediated by an increase in dopamine storage in MN9D cells is provided by immunoblots of chromogranin B, an acidic soluble protein stored within secretory vesicles (Winkler and Fischer-Colbrie, 1992). MN9D cells treated with 124 μM oleic acid exhibit an increase in chromogranin B expression of approximately 3.4-fold (versus DMSO control) and cells exposed to UF show a 1.8-fold increase (versus PBS control)(Fig. 3). Recent microarray data also suggest that there is increased gene expression of secretogranin II, another vesicular soluble protein (Fisher-Colbrie et al., 1995), in sodium oleate-treated MN9D cells as compared to vehicle control. Experiments are in the progess using immunoblotting and immunocytochemical techniques to determine whether there is an increase in secretogranin II protein, in addition to other vesicular markers following exposure to sodium oleate. If the case, this would provide support for a unique mechanism underlying the effect of oleic acid in that the fatty acid-induced elevation in MN9D dopamine content is due to an increase in vesicular storage capacity rather than neurotransmitter synthesis.

Figure 3

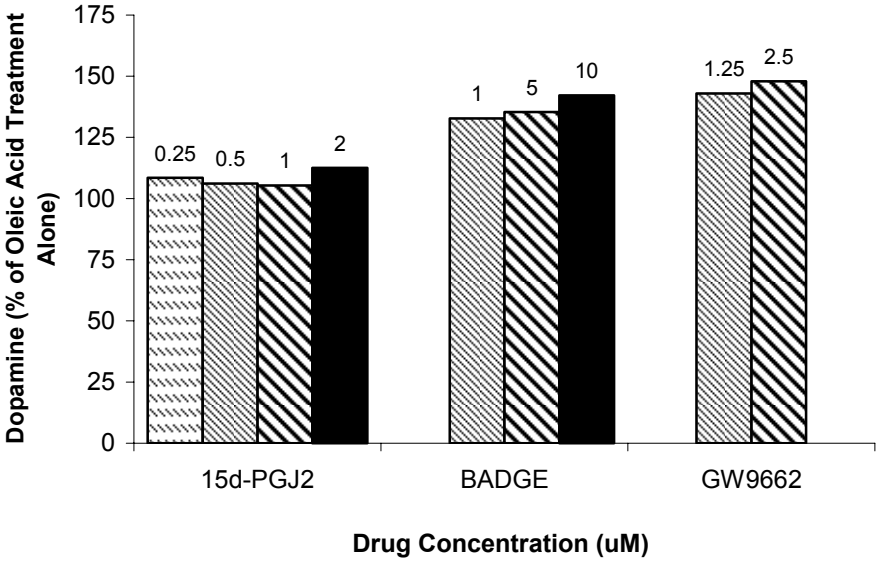


Signaling Mechanisms Underlying the Oleic Acid Induced Increase in MN9D Dopamine

Studies have been initiated examining signaling mechanisms involved in the dopaminergic stimulatory effect of oleic acid. It has been well documented that long-chain, unsaturated fatty acids serve as ligands for several nuclear hormone receptors including peroxisome proliferator-activated receptors (PPAR), retinoic acid (RAR) and retinoid (RXR) receptors, as well as liver X receptors (LXR)(Goldstein et al., 2003; Khan et al., 1992; Lengqvist et al., 2004). Nuclear hormone receptors are ligand-activated transcription factors that can directly regulate gene expression and can affect a wide variety of cellular functions. We therefore investigated whether the dopaminergic stimulatory effect of oleic acid on MN9D cells could be mediated by nuclear receptors.

It has recently been reported that PPAR-γ can regulate neuronal differentiation as well as the dopaminergic phenotype (Park et al., 2004). Exposure of cultured embryonic midbrain cells to 15d-PGJ_2, a PPAR-γ agonist, promotes morphological differentiation of neurons and increases tyrosine hydroxylase expression in such cultures. We examined whether the effect of oleic acid on MN9D dopamine content was mediated by PPAR-γ by treating MN9D cells for 48 hrs with PPAR-γ agonist or antagonist in the presence or absence of 124 μM oleic acid. Exposure of MN9D cells to 0.25 – 2 μM of 15d-PGJ_2, a PPAR-γ agonist, did not affect MN9D dopamine in the presence of oleic acid (Fig 4) or in its absence (data not shown). In contrast, exposure of MN9D cells to oleic acid in conjunction with PPAR-γ antagonists (BADGE or GW9662) resulted in potentiation of MN9D dopamine (up to approximately 45%), above that produced by oleic acid alone (Fig. 4). Neither PPAR-γ antagonist affected dopamine levels of MN9D cells treated with DMSO vehicle (data not shown).

Figure 4



Unsaturated fatty acids are also ligands for RAR and RXR nuclear receptors which are involved in the transduction of retinoic acid signaling. Oleic acid as well as the polyunsaturated fatty acid, docosahexaenoic acid, have been found to bind to RXR and could compete with endogenous retinoic acid for ligand binding (Lengqvist et al. 2004). To examine whether RAR and/or RXR are involved in the dopaminergic stimulatory effect of oleic acid, MN9D cells were treated for 48 hrs in combination with either all-trans retinoic acid (ATRA) or 9-cis-retinoic acid (9-cis-RA). RARs are activated by both ATRA and 9-cis-RA while RXRs primarily respond to 9-cis-RA. As seen in Figs. 5 & 6, ATRA and 9-cis-RA produce a comparable dose-dependent decrease in the ability of oleic acid to elevate MN9D dopamine resulting in an approximate 45-50% inhibition at 10 μM. Exposure of MN9D cells to higher concentrations of ATRA or 9-cis-RA either alone or in combination with oleic acid were toxic (data not shown).

Figure 5

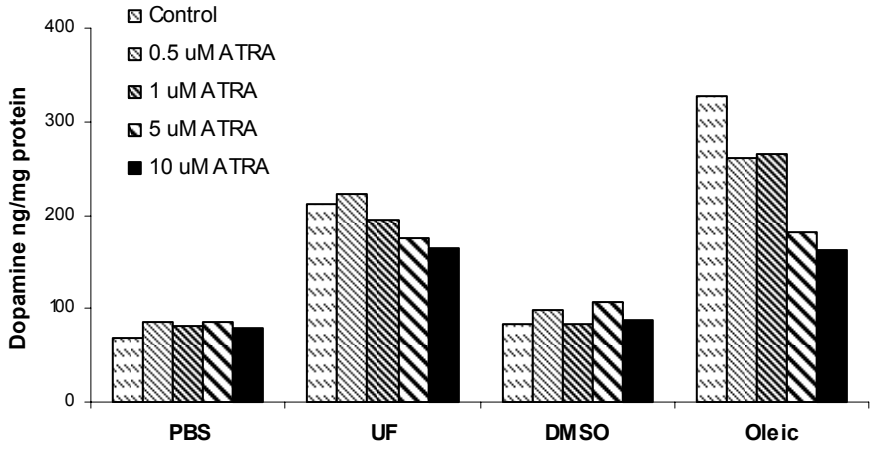
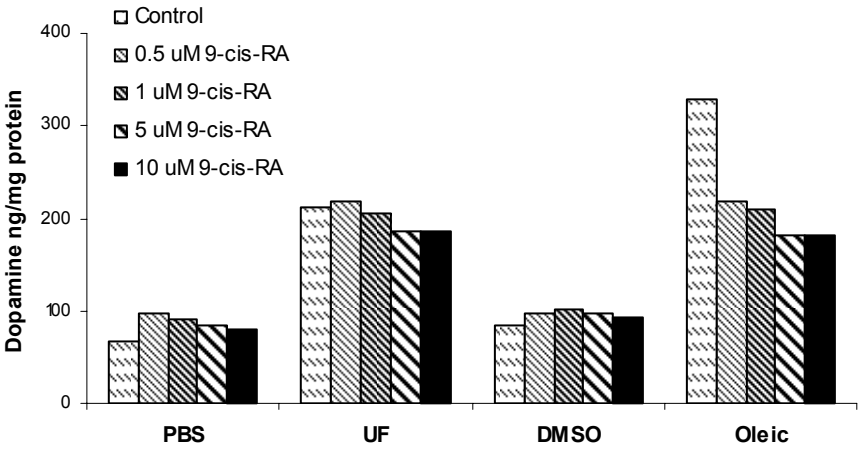
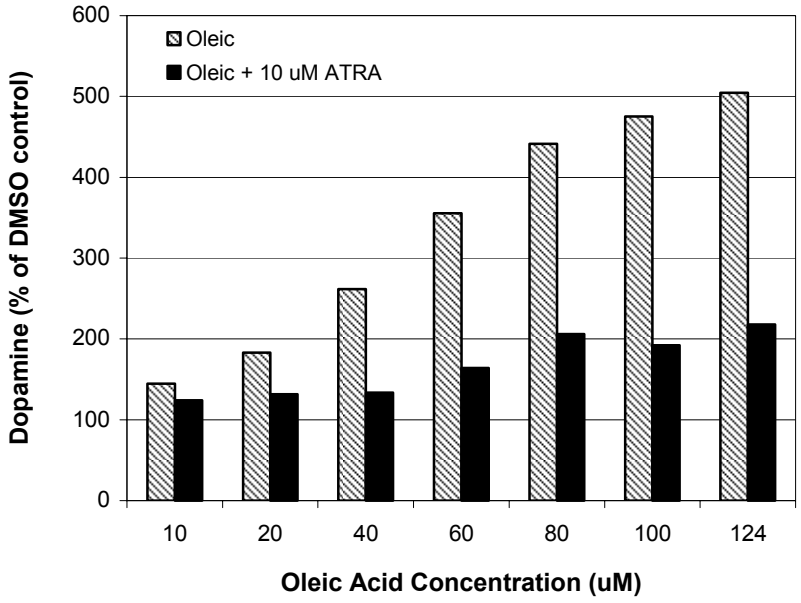


Figure 6



We investigated whether oleic acid could possibly be competing with ATRA for RAR binding sites by varying the concentration of oleic acid and maintaining ATRA at a subtoxic level of 10 μM. Oleic acid produced a dose-dependent increase in MN9D dopamine as compared to DMSO vehicle control (45%-404%)(Fig. 7). Concurrent treatment with 10 μM ATRA only partially blocked (46% inhibition) the dopaminergic stimulatory effect of the lowest, equimolar, concentration of oleic acid tested (10 μM). ATRA was more effective at preventing the oleic acid induced increase in dopamine when MN9D cells were exposed to higher concentrations of the fatty acid. If oleic acid and ATRA simply competed for the same RAR binding site, one would anticipate that 10 μM ATRA would be more effective at inhibiting the dopaminergic stimulatory effects of lower as opposed to higher concentrations of oleic acid.

Figure 7



ATRA is unable to block completely the effects of even the lowest concentration (10 μM) of oleic acid tested. The results suggest that alternative mechanisms are involved in the dopaminergic stimulatory effect of oleic acid and these might include interacting with other nuclear receptors.

RXRs are a common heterodimerization partner for other nuclear receptors. Fatty acid activation of RXR-nuclear receptor heterodimers could regulate the dopaminergic phenotype. For instance, RXR forms heterodimers with Nurr1, an orphan nuclear receptor important for the development of mesencephalic dopaminergic neurons (Zetterstrom et al., 1997). It has been reported that docosahexaenoic acid increases the survival of primary dopaminergic neurons in culture by activation of RXR-Nurr1 heterodimers (Wallen-Mackenzie et al., 2003).

The preliminary results, to date, indicate that nuclear hormone receptor signaling, at least via RAR, is involved in the dopaminergic stimulatory effect of oleic acid on MN9D cells. It is of interest that MN9D dopamine levels can be elevated above that of oleic acid alone by antagonists of PPAR-γ receptors. This finding suggests that the binding of oleic acid to PPAR-γ receptors has an inhibitory effect on the dopaminergic phenotype which can be reversed by PPAR-γ antagonists. The participation of other nuclear hormone receptors in the oleic acid-induced elevation of MN9D dopamine remains to be elucidated.

In addition to regulation by nuclear receptors, studies have also been undertaken to examine whether various protein kinases are involved in the dopaminergic stimulatory effect of oleic acid on MN9D cells. Protein kinase C (PKC) consists of a family of serine/threonine kinases, composed of at least 12 isoforms, which are regulated by a variety of lipid second messengers (Nishizuka, 1995; Toker, 1998). The members of the PKC family have been divided into 3 classes: 1) conventional (sensitive to calcium, diacyglycerol and phosphatidylserine); 2) novel (insensitive to calcium, sensitive to diacylglycerol and phosphatidylserine); and 3) atypical (insensitive to calcium and diacylglycerol, sensitive to phosphatidylserine). Cis-unsaturated fatty acids, such as oleic acid, are known to activate PKC enzymes of all three classes (Asaoka et al., 1992; Khan et al., 1992; Sekiguchi et al., 1987). Inhibitors of conventional (bisindolylymaleimide I), novel (rottlerin) and atypical (PKCζ pseudosubstrate inhibitor) PKC enzymes were tested for their ability to antagonize the oleic acid-induced increase in MN9D dopamine, however none of these agents were able to specifically inhibit the dopaminergic stimulatory effect of this unsaturated fatty acid (data not shown).

Results obtained from experiments using pharmacological inhibitors from other classes of protein kinases including protein kinase A (KT5720), mitogen-activated protein kinases (U0126, SB 203580), tyrosine kinase (Genistein) and phosphatidylinositol 3-kinase (LY 294002) indicate that these protein kinases are relatively ineffective at blocking the elevation of MN9D dopamine by oleic acid (data not shown).

The protein kinases examined thus far do not appear to represent a major signaling pathway for the dopaminergic stimulatory effect of oleic acid. Therefore, we have recently undertaken gene expression analysis in MN9D cells treated with sodium oleate in order to identify key genes related to additional signaling pathways affected by oleic acid and determine its molecular mechanism of action in terms of elevating dopamine. For this purpose, MN9D cells were treated with 124 μM sodium oleate for 24 hrs which resulted in a 3.4-fold increase in cellular dopamine as compared to water (vehicle control). Total RNA was isolated from the cells, synthesized into cRNA which was then hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 array.

A list of up- or down-regulated genes involved in signal transduction as well as other cellular functions exhibiting a difference in expression of at least 2-fold is shown in Tables 5 and 6. Among the cell signaling genes showing up-regulated expression is tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (Ywhaz) also known as 14-3-3ζ. 14-3-3 proteins are binding partners for a large number of cellular proteins involved in diverse cellular processes including signal transduction, cell cycle, transport, apoptosis, development, cell adhesion and transcription (for review, see Fu et al., 2000; van Heusden, 2005). Experiments are in progress using RNA interference methods to determine whether 14-3-

3ζ mediates signaling of the dopaminergic stimulatory effect of oleic acid on MN9D cells. We will determine whether oleic acid is still capable of elevating dopamine in MN9D cells stably transfected with small interfering (si)RNA for 14-3-3ζ. Microarray data from oleate-treated MN9D cells also indicates that this fatty acid modulates the expression of genes involved in various other signaling pathways. Increased expression of Rho GTPase activating protein 12 (Arhgap12) and regulation of G-protein signaling 2 (Rgs2), both of which are guanosine triphosphatase (GTPase)-activating proteins, suggests involvement of G-protein linked signaling (Bernards and Settleman, 2004; Kehrl and Sinnarajah, 2002). There is also down-regulation of Rab39b and Rhob which belong to the Ras superfamily GTPases, molecular regulators of numerous biological functions including vesicular trafficking in secretory and endocytic pathways (Wennerberg et al., 2005; Zerial and McBride, 2001).

Although our initial focus will be on genes involved in signal transduction pathways, microarray analysis has also provided identification of other candidate genes in various functional categories for further investigation into the mechanism of the oleic acid-induced elevation of MN9D dopamine. Of particular interest is the finding that exposure to oleate down-regulates the expression of synapsin I (Syn1), a presynaptic phosphoprotein associated with synaptic vesicles and maintaining a supply of synaptic vesicles available for exocytosis (Humeau et al., 2001; Huttner et al., 1983) and synaptotagmin (Syt4) a postsynaptic protein involved in vesicular trafficking and synaptic plasticity (Yoshihara et al., 2005). Decreased expression of both Syn1 and Syt4 suggests that there is a reduction in neurotransmitter secretion. In contrast, treatment of MN9D cells with oleate results in up-regulated expression of the soluble granular storage protein, secretogranin II (Scg2), supporting our neurochemical findings that the oleate-induced elevation in MN9D dopamine is due to an increase in storage of neurotransmitter which may accumulate if there is less released.

Quantitative RT-PCR will be conducted to confirm transcripts which show marked up- or down-regulation on the DNA microarray as a consequence of exposure to oleic acid. As changes in gene expression are not necessarily accompanied by changes in protein level, Western blot analyses will be conducted, if antibodies are available, to characterize the extent to which the protein product of candidate genes is altered. In order to determine whether differentially expressed genes are specifically involved in signaling pathways underlying the dopaminergic stimulatory effect of oleate, MN9D cells will be transfected with siRNA of candidate genes that show up-regulation by oleate. As described above for 14-3-3ζ, transfected cells will be tested for the ability of oleic acid to increase cellular dopamine. Similarly, genes exhibiting down-regulation by oleate will be overexpressed in MN9D cells and the transfected cells assayed in terms of cellular dopamine response to oleic acid. These studies involving differential gene expression will be conducted in collaboration with Dr. Mitchel Villereal in our Department who has experience with such molecular techniques.

Microarray expression profiling offers an approach to the identification of genes that are differentially expressed following exposure to oleate. The data obtained from such analysis will provide clues as to the molecular mechanisms and signaling pathways underlying the elevation of cellular dopamine by this fatty acid.

Table 5

Genes That Are Significantly Up-Regulated by at Least Two-Fold Following 24 hour Exposure to Sodium Oleate

UP-REGULATED GENES	Accession Number	Gene Symbol	Gene Name
Signal Transduction	AF215668	Adrbk1	Adrenergic receptor kinase, beta 1
	BG064038	Arhgap12	Rho GTPase activating protein 12
	D86352	Casp3	Caspase3
	NM_010517	Igfbp4	Insulin-like growth factor binding protein 4
	L20048	Il2rg	Interleukin 2 receptor, gamma chain
	NM_01090942	Nsg1	Neuron specific gene family member 1
	BB729869	Pask	PAS domain containing serine/threonine kinase
	AI507382	Pkn2	Protein kinase N2
	BM217066	Prkaa1	Protein kinase, AMP activated, alpha 1 catalytic subunit
	AF215668	Rgs2	Regulation of G-protein signaling 2
	BB753533	Shc1	Src homology 2 domain-containing transforming protein C1
	BB828014	Trp53	Transformation related protein 53
	BG968403	Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
Metabolism	X61397	Car8	Carbonic anhydrase 8
	BC006668	Crat	Carnitine acetyltransferase
	NM_016893	Fut8	Fucosyl transferase 8
	BM217066	Galnt1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1
	BG094874	Mlstd2	Male sterility domain containing 2
	BG75139	Mtap	Methylthioadenosine phosphorylase
	AK003786	Nfs1	Nitrogen fixaton gene 1
	BB539054	Pafah1b2	Platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit
	BC003761	Ptp4a1	Protein tyrosine phosphatase 4a1
	BC016252	Txndc5	Thioredoxin domain containing 5
	AK018443	Ube3a	Ubiquitin protein ligase E3A
Transport	NM_007834	Dscr3	Down syndrome critical region gene 3
	BG073833	Nup155	Nucleoporin 155
	AK015412	Kcnab1	Potassium voltage-gated channel, shaker-related subfamily, beta member 1
	U31908	Kcnab2	Potassium voltage-gated channel, shaker-related subfamily, beta member 2
	NM_011256	Pitpnm2	Phosphoinositide transfer protein membrane associated 2
	X69698	Slc2a3	Solute carrier family 2 (facilitated glucose transporter), member 3
	BC003736	Trappc3	Trafficking protein particle complex 3

Table 5, continued

UP-REGULATED GENES	Accession Number	Gene Symbol	Gene Name
Cell Cycle	BF538435	Ccnt1	Cyclin T1
	AV273804	Cul3	Cullin 3
	AW547050	Gas5	Growth arrest specific 5
	BB699415	Mcm2	Minichromosome maintenance deficient 2 mitotin
	BI658327	Mcm3	Minichromosome maintenance deficient 3
	AK010365	Mcm8	Minichromosome maintenance deficient 8 mitotin
	BB706079	Plk4	Polo-like kinase 4
	BI689183	Sep7	Septin 7
	BI684556	Smc211	SMC2 structural maintenance of chromosomes 2-like 1
Transcription	NM_008098	Mtpn	Myotrophin
	BE852433	Phtf2	Putative homeodomain transcription factor 2
	BM932452	Scoc	Short coiled-coil protein
	D50416	Six4	Sine oculis-related homeobox 4 homolog
	BB041813	Srfbp1	Serum response factor binding protein 1
	AF071310	Tnrc11	Trinucleotide repeat containing 11 (THR-associated protein)
	NM_009501	Vax1	Ventral anterior homeobox containing gene 1
	BB667600	Zdhhc20	Zinc finger, DHHC domain containing 20
Development & Differentiation	BC027286	Aggf1	Angiogenic factor with G patch and FHA domains 1
	AV329519	Enah	Enabled homolog (Drosophila)
	AK006840	Fmip	Fms interacting protein
	AV032347	Nif3l1	Ngg1 interacting factor 3-like 1 (S. pombe)
	NM_009035	Rbpsuh	Recombining binding protein suppressor of hairless (Drosophila)
Cellular Organization & Biogenesis	AK013376	Aplp2	Amyloid beta (A4) precursor-like protein 2
	AK012827	Pex3	Peroxisomal biogenesis factor 3
Cellular Component	BG70109	Lamb1-1	Laminin B1 subunit 1
	NM_009129	Scg2	Secretogranin II
	NM_053082	Tspan4	Transmembrane 4 superfamily member 7

Table 6

Genes That Are Significantly Down-Regulated by at Least Two-Fold Following 24 hour Exposure to Sodium Oleate

DOWN-REGULATED GENES	Accession Number	Gene Symbol	Gene Name
Signal Transduction	NM_009652	Akt1	Thymoma viral proto-oncogene 1
	BG064669	Camsap1	Calmodulin regulated spectrin-associated protein 1
	AW986054	Ifi35	Interferon-induced protein 35
	AW121498	Nos3	Nitric oxide synthase 3
	BC024398	Nkiras2	Nf kappa beta inhibitor interacting RAS-like protein 2
	AV162168	Rab39b	Rab39b,member RAS oncogene family
	BC018275	Rhob	Ras homolog gene family, member B
	BM508396	Rtn4rl1	Reticulon 4 receptor-like 1
	AV228782	Sipa1l2	Signal-induced proliferation-associated 1 like 2
	NM_013749	Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a
	AI427806	Usp22	Ubiquitin specific protease 22
Metabolism	NM_019811	Acas2	Acetyl-Coenzyme A synthetase 2 (ADP forming)
	BI413218	Acsl1	Acyl-CoA synthetase long-chain family member 1
	BC019483	Cth	Cystathionase (cystathionine gamma-lyase)
	BB547934	Entpd3	Ectonucleoside triphosphate diphosphohydrolase 3
	NM_019699	Fads2	Fatty acid desaturase 2
	BM235658	Fads3	Fatty acid desaturase 3
	BB028312	Fdft1	Farnesyl diphosphate farnesyl transferase 1
	BC027438	Gale	Galactose-4-epimerase, UDP
	BB188199	Ganab	Alpha glucosidase 2, alpha neutral subunit
	AK013138	Gcat	Glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase)
	NM_010497	Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble
	BB209438	Mical1	Microtubule associated protein monoxygenase calponin and LIM domain containing 1
	BC010318	Pck2	Phosphoenolpyruvate carboxykinase 2
	BI713896	Pmvk	Phosphomevalonate kinase
	C85630	Ppm1a	Protein phosphatase 1A, magnesium dependent, alpha isoform
	BC016535	Uckl1	Uridine-cytidine kinase 1-like 1

Table 6, continued

DOWN-REGULATED GENES	Accession Number	Gene Symbol	Gene Name
Transport	NM_011334	Ccln4-2	Chloride channel 4-2
	AK009435	Ccln7	Chloride channel 7
	C76103	Sec63	SEC63-like (S. cerevisiae)
	AV344473	Snag1	Sorting nexin associated golgi protein 1
	BC022954	Syn1	Synapsin I
	AV336547	Syt4	Synaptotagmin 4
Transcription	AK016820	Aatf	Apoptosis antagonizing transcription factor
	NM_007496	Atbf1	AT motif binding factor 1
	AF375476	Atf5	Activating transcription factor 5
	BC027558	Bola1	BolA-like 1 (E. coli)
	NM_007837	Ddit3	DNA-damage inducible transcript 3
	NM_007913	Egr1	Early growth response 1
	BB524597	KLf7	Kruppel-like factor 7 (ubiquitous)
	NM_017463	Pbx2	Pre B-cell leukemia transcription factor 2
	BG083485	Sox4	SRY-box containing gene 4
	BC012955	Trib3	Tribbles homolog 3 (Drosophila)
	AV305132	Zdhhc6	Zinc finger, DHHC domain containing 6
Cellular Component	NM_025446	Aig1	Androgen-induced 1
	BQ174638	Ank2	Ankyrin 2, brain
	BB150886	Dst	Dystonin
	AW538200	Flnb	Filamin, beta
	BC011306	H2-D1	Histocompatibility 2, K region
	NM_008478	L1cam	L1 cell adhesion molecule
	BF658789	Leprotl1	Leptin receptor overlapping transcript-like 1
	BM942047	Lrrc8	Leucine-rich repeat-containing 8
	BC010596	Map1lc3a	Microtubule-associated protein 1 light chain 3 alpha
	NM_019738	Nupr1	Nuclear protein 1
	NM_013639	Prph1	Peripherin 1
	AY043275	Prima1	Proline rich membrane anchor
	AA762498	Rps9	Ribosomal protein S9

Effect of Oleic Acid on Primary Monoaminergic Neurons Using Three-Dimensional Reaggregate Culture

The stimulatory activities derived from the X61 lysate have been routinely assayed during purification by use of a dopaminergic monoclonal cell line, MN9D. The MN9D cell line permits identification of fractions capable of increasing cellular content of dopamine. The dopamine cell-saving capacity of the fractions, however, requires study of their effects on primary dopaminergic neurons. One of the most effective and flexible methods for studies on primary neurons is three-dimensional reaggregate culture, a system in which dopaminergic, serotonergic and cholinergic neurons can be cultured both in the presence and absence of appropriate target cells (Hsiang et al., 1987; Hsiang et al., 1988; Heller et al., 1992; Heller et al., 1993). In the presence of target cells, essentially all of the dopaminergic neurons survive in reaggregate culture and form extensive axonal arbors which have been shown to selectively innervate the target cells of the corpus striatum (Won et al., 1989; Heller et al., 1997). In the absence of targets or when co-cultured with non-target cells (i.e., optic tectum), no axonal arbors are formed, and the majority of monoaminergic neurons disappear, presumably secondary to cell death. We have already reported on the effect of the X61 stimulatory factor on dopaminergic neurons in such cultures in terms of increased dopamine content (Heller et al., 2000). In addition, mesencephalic-tectal reaggregates treated with the X61 lysate as well as with a partially purified, low molecular weight ultrafiltrate fraction (<5,000 Da) from this lysate, have greater numbers of intensely stained dopaminergic neurons and processes, demonstrating that the X61 active fractions are capable of preventing dopaminergic cell death in the absence of target cells (Won et al., 2003). Given the marked stimulatory effect of oleic acid on raising the cellular dopamine content of MN9D cells, we have examined whether this unsaturated fatty acid is also capable of increasing the cell survival and/or phenotype of primary dopaminergic neurons using reaggregate cultures.

A number of different reaggregate preparations can be utilized to test for activity of specific agents. Mesencephalic cells cultured in the absence of striatal targets show an apparent loss of these monoaminergic neurons and are such preparations are useful for assessing the "cell saving" properties of a given trophic agent. In order to examine effects of oleic acid on monoaminergic cell survival and neurotransmitter levels, mesencephalic-tectal reaggregates were prepared and exposed to dimethylsulfoxide (DMSO, vehicle), 40 μM or 120 μM oleic acid for culture days 1-15. The higher concentration of oleic acid (120 μM) produces a maximal stimulation (~ 4 to 5-fold increase) of MN9D dopamine content, while 40 μM generally results in a 3-fold increase in dopamine. The compounds were replaced every other day along with medium changes. After 15 days of culture, the reaggregates were collected for neurochemical analysis and quantitative assessment of dopaminergic neurons. Serotonin levels were also analyzed since these reaggregate cultures also contain serotonergic neurons of the raphe nuclei. Treatment of mesencephalic-tectal reaggregates with 120 μM oleic acid proved to be toxic after 5 days of exposure and these cultures were not analyzed. As can be seen in Table 7, exposure of these cultures to 40 μM oleic acid during days 1-15 of culture did not effect reaggregate dopamine or serotonin levels nor the number of dopaminergic neurons as assessed by counting tyrosine hydroxylase-immunolabeled cells in 30 randomly selected reaggregate sections from each culture flask.

Table 7

Effect of Oleic Acid on Monoamine Levels and Tyrosine Hydroxylase (TH) Cell Number of Mesencephalic-Tectal Reaggregates

	Dopamine ng/mg protein	Serotonin ng/mg protein	TH Cells
Media alone	8.59 ± 0.27	1.88 ± 0.35	42 ± 7
DMSO vehicle	7.39 ± 0.33	1.39 ± 0.10	30 ± 5
40 μM oleic acid	7.97 ± 0.26	1.93 ± 0.28	27 ± 4

The values represent the mean ± SEM, n = 8 culture flasks.

A similar experiment was conducted in which mesencephalic-tectal reaggregates were treated with sodium oleate, a water soluble salt of oleic acid. Sodium oleate is also capable of increasing MN9D cellular dopamine. Reaggregates were exposed during days 1-15 of culture to 25, 50 or 75 μM sodium oleate and then collected for neurochemical analysis. There was no effect of sodium oleate, at any concentration, on reaggregate dopamine or serotonin levels (data not shown).

The lack of an effect of oleic acid or sodium oleate on monoaminergic neurons from mesencephalic-tectal reaggregates prompted us to test whether this unsaturated fatty acid could protect against neurotoxicity. An *in vitro* model of neuronal damage was employed, that of methamphetamine-induced monoamine depletion of the nigrostriatal pathway. We have previously demonstrated that reaggregates composed of dopamine and serotonergic neurons cultured with their target cells (i.e., mesencephalic-striatal reaggregates) respond to toxicologic agents in a manner similar to that observed *in vivo* (Heller et al., 1993; Kontur et al., 1991; Won et al., 1992). In the case of methamphetamine, this agent produces a marked reduction in reaggregate monoamine levels which persists for at least 3 weeks after the drug has been removed (Won et al., 1992). For the purpose of examining whether sodium oleate has potential neuroprotective activity, 15 day old mesencephalic-striatal reaggregates were exposed to 50 or 100 μM sodium oleate in the presence or absence of 10 μM methamphetamine for 7 days. As seen in Table 8, one week exposure to methamphetamine significantly reduced reaggregate dopamine (-45%, $p<0.001$) and serotonin (-71%, $p<0.001$) levels as compared to water vehicle control. Concurrent treatment with 50 or 100 μM sodium oleate did not reverse methamphetamine-induced monoamine depletion. Seven day exposure to sodium oleate, alone, had no effect on reaggregate dopamine or serotonin levels.

Table 8

Effect of Sodium Oleate on Methamphetamine-Induced Monoamine Depletion of Mesencephalic-Striatal Reaggregates

	Dopamine ng/mg protein	Serotonin ng/mg protein
Water vehicle	31.70 ± 0.77	20.85 ± 0.69
50 μM Sodium Oleate	29.84 ± 0.68	18.22 ± 0.60
100 μM Sodium Oleate	29.63 ± 0.78	19.13 ± 0.16
10 μM Methamphetamine	17.40 ± 0.90*	5.95 ± 0.15*
10 μM Methamphetamine + 50 μM Sodium Oleate	13.81 ± 0.84	5.02 ± 0.24
10 μM Methamphetamine + 100 μM Sodium Oleate	16.92 ± 0.47	6.21 ± 0.26

The values represent the mean ± SEM, n = 4 culture flasks.
*Significantly different from water vehicle control, $p<0.001$.

Given the marked stimulatory effect of oleic acid or sodium oleate on MN9D cellular dopamine content, it was puzzling that this unsaturated fatty acid lacked a similar effect on primary dopaminergic neurons. Since the initial reaggregate experiments were conducted using culture medium containing horse serum, it was possible that fatty acids in the serum were present at levels sufficient to maintain the dopaminergic phenotype. Serum-free, defined medium circumvents the possible contribution of endogenous factors present in serum and is commonly used in assessing effects of trophic agents in culture preparations.

To test whether any effects of oleic acid on primary monoaminergic neurons may have been masked by maintaining the cultures in serum-containing medium, mesencephalic-striatal reaggregates were prepared and initially grown in serum-containing medium for 6 days and then cultured for an additional 9 days in defined medium with N2.1 supplements (Bartlett and Banker, 1984) in the presence of 2.9 mg/ml fatty acid-free bovine serum albumin (BSA), 10 μM linoleic acid-BSA, and/or, 75 μM oleic acid-BSA. This formulation of defined medium does not contain exogenous serum proteins which serve as fatty acid carriers. Since high concentrations of free fatty acid can be toxic to cells, linoleic acid and oleic acid were added to media in the form of

BSA conjugates. Linoleic acid, an essential fatty acid involved in structural membrane integrity and cell growth, was used at a concentration in the physiological range of levels normally present in serum.

It is apparent that a small, but significant effect of oleic acid on dopamine (+30%, $p<0.05$) and serotonin (+40%, $p<0.005$) levels is revealed when mesencephalic-striatal reaggregates are cultured in defined medium (Table 9). Exposure to 10 μM linoleic acid for 9 days also has a stimulatory effect on reaggregate monoamine content resulting in an increase in dopamine (+71%, $p<0.005$) and serotonin (+69%, $p<0.005$) levels as compared to BSA vehicle control. Previous structure-activity studies of the effect of unsaturated long-chain fatty acids demonstrated that linoleic acid, in addition to oleic acid, was effective at raising MN9D cellular dopamine (see Annual Report of December 2004). The concurrent administration of linoleic and oleic acids also enhances reaggregate dopamine (+62%) and serotonin (+65%) levels, however the effect of both fatty acids is not additive.

Table 9

Effect of Oleic Acid and/or Linoleic Acid on Monoamine Levels of Mesencephalic-Striatal Reaggregates Cultured in Serum-Free Medium

	Dopamine ng/mg protein	Serotonin ng/mg protein
BSA vehicle	3.15 ± 0.21	2.68 ± 0.11
75 μM Oleic-BSA	4.10 ± 0.29*	3.76 ± 0.22**
10 μM Linoleic-BSA	5.40 ± 0.37**	4.53 ± 0.10**
10 μM Linoleic-BSA + 75 μM Oleic-BSA	5.09 ± 0.34**	4.42 ± 0.15**

The values represent the mean ± SEM, n = 6 culture flasks.
*Significantly different from BSA vehicle control, $p<0.05$.
**Significantly different from BSA vehicle control, $p<0.005$.

The finding that oleic acid and linoleic acid are capable of elevating both dopamine and serotonin levels in mesencephalic-striatal reaggregates is important and clearly warrants further study as to the selectivity of effects of these fatty acids with respect to various cell phenotypes.

Since a stimulatory effect of oleic acid could be observed on monoaminergic neurons when such cells were grown with target cells in the presence of defined media, we conducted a similar experiment using mesencephalic-tectal reaggregates to determine whether oleic acid stimulated

the monoaminergic phenotype when such cells were cultured in the absence of target. Mesencephalic-tectal cultures were exposed to 75 μM oleic-BSA, 10 μM linoleic acid or a combination of both fatty acids during culture days 6-15. In the absence of target cells, oleic and linoleic acids, alone or in combination, had no effect on reaggregate dopamine level (Table 10). In contrast, serotonin was elevated 65% by linoleic acid ($p<0.005$) and showed a moderate 25% increase by oleic acid ($p<0.05$). Exposure to both fatty acids did not result in a significant effect on reaggregate serotonin.

Table 10

Effect of Oleic Acid and/or Linoleic Acid on Monoamine Levels of Mesencephalic-Striatal Reaggregates Cultured in Serum-Free Medium

	Dopamine ng/mg protein	Serotonin ng/mg protein
BSA vehicle	3.87 ± 0.18	1.88 ± 0.11
75 μM Oleic-BSA	4.41 ± 0.19	2.35 ± 0.07*
10 μM Linoleic-BSA	4.46 ± 0.21	3.10 ± 0.26**
10 μM Linoleic-BSA + 75 μM Oleic-BSA	4.94 ± 0.41	2.46 ± 0.24

The values represent the mean ± SEM, n = 5 culture flasks.
*Significantly different from BSA vehicle control, $p<0.05$.
**Significantly different from BSA vehicle control, $p<0.005$.

Linoleic and oleic acid are less capable of elevating dopamine in mesencephalic-tectal as compared to mesencephalic-striatal reaggregates suggesting that target-derived substances, in addition to factors contained within serum, are important for maintaining the dopaminergic phenotype.

It is apparent from these studies using three-dimensional reaggregate cultures that serum contains levels of fatty acids, as well as other factors, which are sufficient for maintenance of the monoaminergic phenotype. A stimulatory effect of exogenous oleic acid on primary monoaminergic neurons is only unmasked when the reaggregates are cultured in a serum-free, defined medium. These preliminary experiments indicate that serum-free defined medium

containing N2.1 supplements is not optimal for maintaining dopaminergic neurons in reaggregate culture because dopamine concentrations of control cultures grown in serum-free medium were at least 50% less as compared to reaggregates grown in 10% horse serum. In addition, light microscopic examination of sections from reaggregates cultured in serum-free medium and stained with cresyl violet showed that the cultures contained a considerable number of pyknotic profiles (data not shown). The reduction of cell viability within the reaggregates may be due to the fact that the switch from serum-containing to serum-free medium within a single medium exchange may have been too abrupt. Therefore, it may be necessary to adapt the cultures to the absence of serum through a series of progressive dilutions of serum over successive media exchanges. Furthermore, although N2.1 supplemented medium was used in these initial studies, alternative serum-free defined media need to be explored with respect to improving the growth and viability of reaggregate cultures. More recent serum-free formulas are now commercially available such as Neurobasal medium in combination with B27 supplements which has been demonstrated to support long-term culture and differentiation of embryonic neurons from a number of brain regions including the substantia nigra, as well as adult rat hippocampal neurons (Brewer, 1995; Brewer, 1997). Manipulation of the culture conditions, as described above, should improve reaggregate viability allowing for better assessment of the effect of oleic acid on primary monoaminergic neurons in terms of phenotypic expression and cell survival.

In contrast to oleic acid, the dopaminergic stimulatory effect of a partially purified, low molecular weight (<5,000 Da) ultrafiltrate fraction (UF4) from X61 cell lysate could be observed in mesencephalic-tectal reaggregates cultured in the presence of serum, as evidenced by an increase in dopamine concentration and dopaminergic cell number (Heller et al., 2000; Won et al., 2003). Structural identification of the active molecule(s) in the low molecular weight ultrafiltrate fraction is currently in progress and we intend to utilize the reaggregate culture system to assess the ability of such identified compounds, as they become available, to increase primary dopaminergic function and prevent dopaminergic cell death.

RESEARCH ACCOMPLISHMENTS

- Further purification of a low-molecular weight, hydrophilic dopaminergic stimulatory activity in a YC-05 concentrate fraction from days 2 and 3 autodigested X61 lysate using additional procedures (charcoal adsorption, re-concentration, repeat gel filtration chromatography) to reduce the concentration of free amino acids in the sample. The more purified preparation should permit structural analysis of the molecules responsible for the activity using mass spectroscopy and nuclear magnetic resonance.

- Neurochemical evidence that the elevation of MN9D cellular dopamine by oleic acid as well as a water-soluble, ultrafiltrate fraction involves an increase in vesicular storage as opposed to an increase in neurotransmitter synthesis.

- Evidence supporting an increase in vesicular storage of dopamine from Western blot analysis demonstrating enhanced expression of the secretory vesicle storage marker chromogranin B, in MN9D cells exposed to oleic acid or the water-soluble, ultrafiltrate fraction. In addition, microarray analysis of oleate-exposed MN9D cells indicates an up-regulation of secretogranin II, another secretory vesicle marker associated with catecholamine storage.

- Results demonstrating that nuclear hormone receptors, in particular retinoic acid receptors, play a role in the dopaminergic stimulatory effect of oleic acid.

- Demonstration that a stimulatory effect of oleate on primary dopaminergic neurons co-cultured with their target cells is unmasked when the reaggregate cultures are maintained in serum-free, defined medium as opposed to serum-containing medium.

REPORTABLE OUTCOMES

Published Manuscript (included in the Appendix)

Heller, A., Won, L., Bubula, N., Hessefort, S., Kurutz, J.W., Reddy, G.A., and Gross, M. Long-chain fatty acids increase cellular dopamine in an immortalized cell line (MN9D) derived from mouse mesencephalon, Neuroscience Letters 376, 35-39, 2005.

CONCLUSIONS

Parkinson's disease remains a major world-wide health problem particularly in the aging population. In North America alone, over one million individuals suffer from the disease experiencing severe motor dysfunction with muscle rigidity, bradykinesia and tremor. The motor disabilities are a result of degeneration of a dopaminergic projection arising in the mesencephalon and providing an extensive innervation to the corpus striatum a subdivision of brain subserving extrapyramidal motor function. Therapeutic interventions, therefore, which can prevent or retard dopaminergic cell loss, increase sprouting of dopaminergic axons or upregulate the phenotype of remaining dopaminergic nigrostriatal neurons have the potential for reversing the motor deficits.

Given that Parkinson's disease is a degenerative disorder involving loss of the dopaminergic neurons of the nigrostriatal projection, considerable interest has focused on the possible therapeutic role of trophic agents. We have purified two types of chemically distinct dopaminergic stimulatory factors from the lysate of immortalized, striatal X61 cells. One type of factor could be extracted from the cell lysate by isoamyl alcohol/chloroform and was identified to be composed of primarily oleic acid. The other factor(s), whose structure is currently under investigation, is small and hydrophilic. Our experimental results indicate that both types of dopaminergic stimulatory factors elevate the dopamine content of mesencephalic-derived MN9D cells dopamine through an increase in storage capacity rather than neurotransmitter synthesis. These findings suggest a novel mechanism of action by which such factors regulate the dopaminergic phenotype. The increase MN9D dopamine content by oleic acid is partially mediated by an interaction with nuclear hormone receptors. Identification of additional signaling cascades relevant to the dopaminergic stimulatory effect of oleic acid is underway utilizing cDNA microarrays to analyze differentially expressed genes. The availability of unique trophic factors, as well as understanding their mechanism of action, could provide novel strategies for neuroprotective therapy that might slow disease progression or restore function in neurodegenerative disorders such as Parkinson's Disease.

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Long-chain fatty acids increase cellular dopamine in an immortalized cell line (MN9D) derived from mouse mesencephalon

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Abstract

The lysate of an immortalized monoclonal cell line derived from the striatum (X61) contains a dopaminergic stimulatory activity that is capable of increasing the dopamine content of an immortalized mouse mesencephalic cell line (MN9D) which expresses a dopaminergic phenotype. Purification of an isoamyl alcohol extract of this lysate and subsequent identification by NMR spectroscopic analysis demonstrated that the dopaminergic stimulatory activity contained within the lysate was a mixture of 80–90% *cis*-9-octadecenoic acid (oleic acid) and 10–20% *cis*-11-octadecenoic acid (*cis*-vaccenic acid). The effect of oleic acid on MN9D dopamine is a prolonged event. MN9D dopamine increases linearly over a 48 h period suggesting the induction of an increased dopaminergic phenotype in these dividing cells. The ability to increase MN9D dopamine by oleic and *cis*-vaccenic acids is shared by a number of other long-chain fatty acids including arachidonic, linoleic, linolenic, palmitoleic, and *cis*-13-octadecenoic acid. The possibility that oleic or other relatively innocuous fatty acids might affect dopaminergic function in primary neurons is intriguing with respect to possible therapeutic approaches to the treatment of dopaminergic cell loss and the motor sequelae of Parkinson's disease.

Keywords: Dopamine; Fatty acids; MN9D; Nigrostriatal; Parkinson's disease; Trophic factor

Immortalized monoclonal cells of the mouse nigrostriatal projection have been developed as an approach to the identification of substances which could regulate dopaminergic function and cell survival [1,14]. The dopaminergic MN9D cell line of mesencephalic origin and the X61 cell line of striatal origin were obtained by somatic cell fusion with the N18TG2 neuroblastoma which is lacking the hypoxanthine phosphoribosyltransferase enzyme [1,14]. We have previously demonstrated that striatal-derived monoclonal cells (X61) contain dopaminergic stimulatory substances which increase the dopamine content of MN9D cells [3]. Striatal cell lines (X61) provide a source for such substances and the mesencephalic-derived MN9D cell line provides a rapid assay method for detecting active molecules capable of modulating cellular dopamine. The crude cell lysate of X61 cells, as well as a partially purified ultrafiltrate preparation (UF4) of that lysate, also increases the dopamine content of primary dopaminergic neurons grown in reaggregate culture in the absence of target cells (i.e., mesencephalic cells co-cultured with tectum, a non-target region for dopaminergic neurons) as well as levels of homovanillic acid in the culture medium [15]. In such cultures, in which the majority of dopaminergic neurons are lost due to the absence of target cells, treatment with the crude lysate or UF4 ultrafiltrate results in a 2- (UF4) to 2.9- (X61 lysate) fold increase in the density of dopaminergic neurons in the treated cultures [15].

The UF4 ultrafiltrate contains active substances, probably peptides, of low molecular weight and high water solubility. It was, however, apparent that the bulk (two-thirds) of

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dopaminergic stimulatory activity present in the processed X61 cells resided in a fraction which did not pass through a YM-5 ultrafiltration membrane and was lipid soluble. The present study was conducted to determine the chemical nature and activity of this major fraction.

The UF4 ultrafiltrate was obtained from sonicated lysates of X61 cells which were allowed to "autodigest" at room temperature for 2 days and concentrated by pressure filtration through an Amicon YM-5 membrane (5000 Da molecular weight cut-off). Some form of digestion occurs in this process as evidenced by the fact that 2 days of incubation at room temperature results in the conversion of some (approximately 30%) of the dopaminergic stimulatory activity in the X61 cell lysate from a high molecular weight form to a size that can pass through a YM-5 membrane (see [15] for details).

The low molecular weight UF4 ultrafiltrate fraction contained significant dopaminergic stimulatory activity as assessed by effects on MN9D cells. However, the majority of the activity from the "autodigested" X61 cell lysate did not pass through the YM-5 ultrafiltration membrane. Approximately two-thirds of the activity resided in the material remaining on the high molecular weight side of the Amicon YM-5 membrane and is referred to as "X61 concentrate". This X61 concentrate was subsequently extracted with 2 M NaCl followed by a 1:1 mixture of isoamyl alcohol/chloroform. The isoamyl alcohol/chloroform extract was shown to contain materials capable of increasing MN9D dopamine levels. This activity is not extractable from fresh X61 cell lysate, but appears to require the autodigestion step with time for the activity to become liberated from some cell component and be available for organic extraction.

The isoamyl alcohol/chloroform soluble stimulatory activity was taken up by a C18 reverse phase column from a mixture of 70% acetonitrile/30% (0.05% trifluoroacetic acid in water) and then eluted by a linear gradient from the mixture to 100% acetonitrile. The active fractions from the column showed some absorbance at 215 nm, but the bulk of absorbance was seen in fractions devoid of activity, suggesting that the reverse phase separation resulted in considerable purification of the activity. The active fractions from the reverse phase column were then applied to a Phenomenex 5 μm, 50 Å Phenogel gel filtration sizing column. The dopaminergic stimulatory activity eluted from the Phenogel column in 100% acetonitrile within a single absorbance peak. The Phenogel fractions containing dopaminergic stimulatory activity were subjected to mass spectrographic analysis. Two peaks of high intensity were observed with molecular weights of 283 and 565.

NMR spectroscopy demonstrated that the single elution peak from the Phenogel column contained two chemical moieties, the greater of which constituted approximately 80–90% of the material (Fig. 1). The 600 MHz ^{1}H 1D NMR spectrum of the sample showed one major set of peaks and no significant minor peaks, suggesting a sample purity greater than 95%. The ^{13}C 1D NMR spectrum, however, showed two sets of peaks, indicating that the sample contained two species

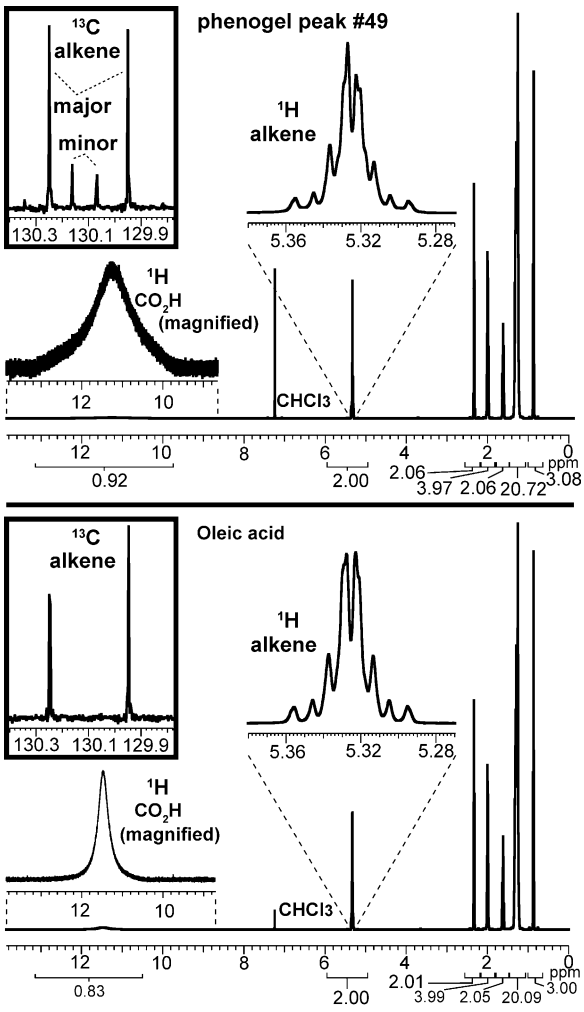

Fig. 1. The NMR spectra of peak #49 from the Phenogel column purified material (top) as compared to commercial oleic acid (bottom). The ^{1}H spectrum is shown at the bottom of each panel, and the peak integral values of the intensities are indicated below the axes. The carboxylic acid and alkene regions of the ^{1}H spectra are magnified and expanded. The alkene regions of the ^{13}C spectra are shown as insets. It is apparent that the Phenogel #49 sample contains one major and one minor species. The minor species was identified by NMR analysis to be *cis*-vaccenic acid.

so closely related that they gave rise to virtually identical ^{1}H NMR spectra. The minor component made up approximately 10–20% of the total sample, according to their relative intensities in the ^{13}C spectra. The positions of the ^{1}H and ^{13}C peaks in these spectra ruled out the possibility that the sample contains protein, peptide, DNA, RNA, carbohydrate, glycolipid, steroid, or other cholesterol-related molecules. The positions are instead consistent with those expected from a fatty acid. Comparison of the major species' spectra with ^{1}H and ^{13}C NMR spectra of various fatty acids showed that they were very similar to those of *cis*-9-octadecenoic acid (oleic acid). It remained uncertain whether the unsaturated bond was of *cis* or *trans* configuration. Pure *cis*-9-octadecenoic acid and *trans*-9-octadecenoic acid (elaidic acid) (Aldrich), and their ^{1}H and ^{13}C NMR spectra were compared to those of the sam-

ple. We found that the major species' peaks were essentially identical to those of the *cis* isomer (i.e., oleic acid) (Fig. 1).

A variety of compounds structurally related to oleic acid were obtained and examined by NMR spectroscopy to determine the identity of the minor compound in the sample. We found that the ^{1}H and ^{13}C NMR spectra of *cis*-vaccenic acid (*cis*-11-octadecenoic acid), which has the same empirical formula as oleic acid, were essentially identical to those of the minor species in the sample. Thus, our NMR data support the conclusion that the purified sample comprises a mixture of approximately 80–90% *cis*-oleic acid and 10–20% *cis*-vaccenic acid.

The NMR spectral analysis with known synthetic entities established the chemical composition of the majority of the purified material to be *cis*-9-octadecenoic acid (oleic acid). The mass spectroscopic analysis is in accord with the NMR data in that the molecular weight of 283 corresponds to that of oleic acid and/or *cis*-vaccenic acid and the larger sized 565 molecular weight species may well represent a dimerization of these long-chain fatty acids.

In order to determine whether the Phenogel purified material and synthetic oleic acid produce similar effects on MN9D cellular dopamine, MN9D cells were plated into six-well culture plates and cultured in Dulbecco's Modified Eagle's medium containing 5% (v/v) Fetal Clone III and 1% (v/v) penicillin–streptomycin (5000 units penicillin/5000 μg streptomycin). The cells were exposed to increasing concentrations of oleic acid or the Phenogel purified material, diluted in dimethylsulfoxide (DMSO), for 48 h and then collected for analysis of cellular dopamine content using high performance liquid chromatography. Protein content of the cultures was determined spectrophotometrically [11].

In agreement with the NMR data, the Phenogel purified material and synthetic oleic acid showed identical concentration–response in terms of increasing MN9D dopamine as shown in Fig. 2. The concentration–response of MN9D cells to oleic acid has been repeated in two other experiments and the results obtained were identical. Exposure of MN9D cells to concentrations of oleic acid or the Phenogel purified material greater than 124 μM produced either less of a dopaminergic stimulatory effect or was actually toxic to the cells. Thus, the response at 124 μM was considered to be maximal. The maximal effect of oleic acid and the Phenogel purified material in this experiment represents an approximate five-fold increase in dopamine level over controls. The EC_{50} for oleic acid is approximately 5.5×10^{-5} M. The effects of oleic acid are not secondary to either an increase in MN9D cell proliferation or differentiation. This issue was tested directly in a separate experiment in which dopamine levels, cell number and the state of cell differentiation were examined. MN9D cells were exposed to DMSO or 124 μM oleic acid for 48 h. An increase in dopamine of 8.3-fold was observed in this experiment. The protein content of MN9D cells exposed to oleic acid was essentially identical to that of the DMSO group and the number of cells in the oleic acid-treated group (0.89 ± 0.13 million cells, mean ± S.E.M., $n = 6$) was not significantly different from DMSO vehicle (1.05 ± 0.10 million cells, mean ± S.E.M., $n = 6$). The oleic acid-exposed cells showed none of the characteristics of differentiated MN9D cells, i.e. a reduction in cell number or increased process outgrowth [1].

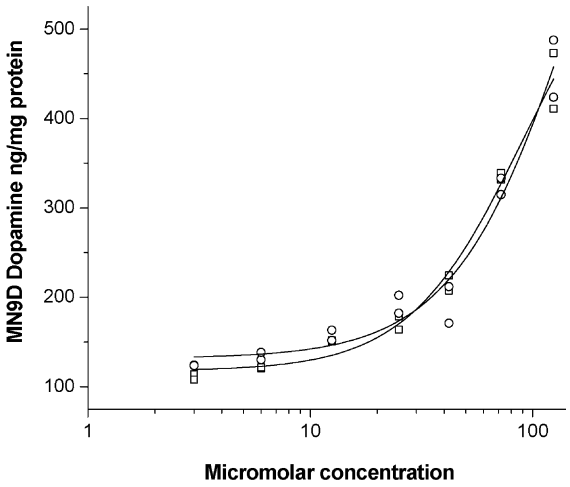

Fig. 2. Effect of 48 h exposure to increasing (log scale) concentrations (3–124 μM) of *cis*-9-octadecenoic acid (oleic acid, squares) or to the Phenogel column purified material from X61 cells (circles) on MN9D dopamine levels. MN9D dopamine level of DMSO vehicle control = 109.9 ng/mg protein.

Given that the assays were carried out in serum-containing medium and the known capacity of oleic acid to bind to serum proteins [8,12], the free EC_{50} for oleic may be considerably lower than this estimate. The level of oleic acid required to increase dopamine levels under serum-free conditions cannot be tested with MN9D cells since they do not grow well under conditions of low serum or serum-free medium. The MN9D cells, in addition, only permit the examination of effects on the catecholaminergic phenotype. The issue of whether oleic acid affects other transmitter phenotypes will require primary neuronal cultures.

While *cis*-vaccenic acid, the minor constituent of the Phenogel purified fraction, is active (see below), a comparison of the concentration–response curve of *cis*-vaccenic with oleic acid on MN9D dopamine, suggests that *cis*-vaccenic acid has a slightly lower potency.

A comparison of the effect of oleic versus the *trans* isomer (elaidic acid), using 11 concentrations ranging from 0.3 to 353 μM, on MN9D dopamine revealed that elaidic acid showed only minimal activity (less than 10% elevation in dopamine even at the highest concentration of 353 μM) (data not shown).

A number of more detailed studies have been initiated on the effect of oleic acid in increasing MN9D dopamine. The first of these studies, a time course on the effect of the Phenogel purified material and oleic acid, demonstrates a linear increase in MN9D dopamine over 48 h (Fig. 3). The effect of both the known compound and the Phenogel purified material in this experiment is impressive, resulting, after 48 h, in at

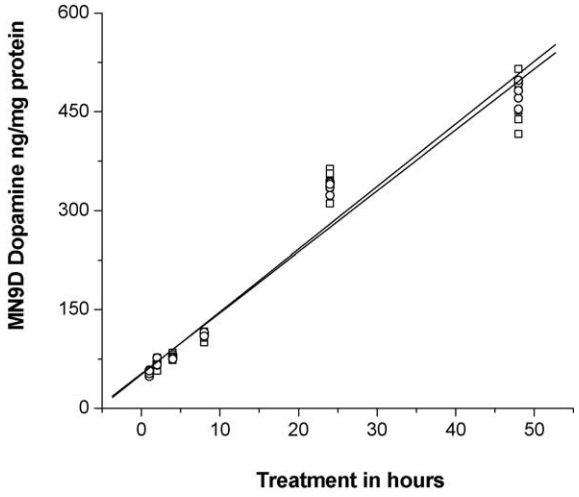

Fig. 3. Time course of the effect of exposing MN9D cells to 124 μM of *cis*-9-octadecenoic acid (oleic acid, squares) or 124 μM of the Phenogel column purified material from X61 cells (circles) on cellular dopamine levels. $N = 6$ cultures per time point. MN9D dopamine level (ng/mg protein) of DMSO vehicle control for the various time points: 1 h, 54.1; 2 h, 63.3; 4 h, 63.6; 8 h, 65.3; 24 h, 77.3; 48 h, 67.5.

least an 8.5-fold increase in MN9D dopamine as was seen in the experiment on cell proliferation described above. Given that the MN9D cells, which are fusion products of mesencephalic cells and the N18TG2 neuroblastoma, are doubling every 24 h, this result would suggest that the effect of the active chemical is to induce an increase in the dopaminergic phenotype of MN9D cells. If that is the case, then the effect should be present in the daughter cells as they appear.

As part of this study, a limited structure–activity analysis was conducted using a variety of common long-chain unsaturated fatty acids. It is clear from this study, that the ability of oleic acid to increase MN9D dopamine content over a 48 h period is shared by a number of other long-chain fatty acids containing one to four double bonds, as shown in Table 1. Concentration–response curves were determined for each of the indicated compounds and compared to oleic acid using seven concentrations over a range of 3–124 μM. The majority of the compounds tested were active, but in most cases, a maximal response was not obtained even at 124 μM. For this reason, Table 1 provides the cellular MN9D dopamine level (ng/mg protein) following 48 h of treatment with 124 μM of each respective compound, and the amount of increase this represents over the DMSO vehicle control.

Table 1
Effect of unsaturated long-chain fatty acids on MN9D cellular dopamine

Compound	Dopamine (ng/mg protein)	Fold-increase over DMSO control
Oleic acid	332	4.8
Arachidonic acid	365	5.3
Linoleic acid	300	4.3
Linolenic acid	297	4.3
Palmitoleic acid	280	4.0
cis-Vaccenic acid	264	3.8
cis-13-Octadecenoic acid	134	1.9
Petroselenic acid	92	1.3
Oleic anhydride	81	1.2

MN9D cells were treated for 48 h with 124 μM of each of the compounds indicated above and then cellular dopamine content determined. All compounds were dissolved in dimethylsulfoxide (DMSO). Dopamine level of DMSO vehicle control = 69 ng/mg protein.

Two of the compounds tested showed only minimal effects: oleic anhydride, formed by the fusion of two molecules of oleic acid with the splitting out of water, and petroselenic acid, an 18 carbon monoenoic acid in which the double bond is at position 6, in contrast to oleic acid with a double bond at position 9. The remaining seven long-chain fatty acids were all active and included both monoenoic acids (oleic, palmitoleic, *cis*-vaccenic, and *cis*-13-octadecenoic acid) and polyenoic acids (linoleic with two double bonds, linolenic with three double bonds and arachidonic with four double bonds).

Thus, it is clear that a variety of long-chain fatty acids are capable of increasing the dopamine content of a mesencephalic-derived immortalized monoclonal cell line expressing a dopaminergic phenotype. While the number and variety of fatty acids tested is too limited to reach systematic conclusions regarding the optimal structure necessary to produce an increase in MN9D dopamine, the ability to significantly elevate MN9D dopamine content appears to depend on the presence of a carboxylic acid group and the relative position of an unsaturated double bond. Both petroselenic and oleic acid are C18 fatty acids. Petroselenic acid, which shows minimal effects (see Table 1), has a *cis* double bond that is located three carbons further from the terminal carboxylic acid than that of oleic acid, thus suggesting that the length of the side chain may be a critical determinant of effect.

The MN9D cell line has served as a useful test object for monitoring the presence and purification of dopaminergic stimulatory activities from lysate of immortalized monoclonal cell lines (X61) derived from the striatum. In addition, at least with respect to the small, water-soluble activity (UF4) which appears to be peptide in nature, effects on the MN9D line were predictive of the ability of this substance to increase the dopamine content of primary dopaminergic neurons and prevent their loss in the absence of striatal targets [15]. Whether the increases in MN9D dopamine seen following treatment with known long-chain fatty acids described here will be replicated on primary dopaminergic neurons is obviously a critical question and is currently being examined using three-dimensional reaggregate culture in a similar manner to the previous studies [3,15].

The striatal lines were developed specifically for the purposes of providing a substantial source of monoclonal cells which could be probed for substances that might influence dopaminergic function, either with respect to dopamine levels, cell survival, or the maintenance of the phenotype. It is worth noting that while an isoamyl alcohol/chloroform extract of a lysate concentrate of the X61 cell yielded a fraction capable of markedly increasing MN9D dopamine, that fraction on purification turned out to contain

long-chain fatty acids (oleic and *cis*-vaccenic acid) which could have been extracted from any number of sources. The presence of these active moieties in the X61 cell line, however, directed our attention to their isolation and purification.

Although this is the first description of an ability of long-chain fatty acids to increase cellular dopamine, there are many reports of effects of oleic and other unsaturated fatty acids on neuronal function. Oleic acid is an activator of protein kinase C activity [4,7] and, as has been reported, its synthesis has been linked to neuronal differentiation during development [6,13] and the promotion of axonal growth and induction of MAP-2 expression (microtubule associated protein-2), a marker of dendritic differentiation [9]. Arachidonic acid markedly stimulates, in a dose-dependent fashion, the spontaneous release of dopamine in purified synaptosomes from rat striatum and inhibits dopamine uptake [5]. In addition, reduced dietary intake of omega-6 (arachidonic acid) and omega-3 (docosahexanoic acid) fatty acids in piglets during the first few weeks of postnatal life has been shown to result in lower brain monoamine concentrations which can be reversed upon supplementation with adequate levels of these fatty acids [2]. We are not aware of any reports of beneficial effects of dietary unsaturated fatty acids on the pathogenesis or clinical course of Parkinson's disease.

While the treatment of the motor sequelae of Parkinson's disease has received intense study for over four decades with some very notable successes [10], it remains clear that additional treatment modalities would be helpful and are being sought. It is intriguing in this regard that a long-chain fatty acid such as oleic acid, which is essentially a benign dietary material, can markedly increase the dopamine content of a cell expressing a dopaminergic phenotype. If it should prove to be the case, that oleic acid or other active fatty acids increase the dopamine content of primary dopaminergic neurons, as we previously reported for the crude X61 lysate and partially purified ultrafiltrates (UF4), the long-chain fatty acids may provide an interesting addition to pharmacological approaches to the investigation of the disease and its treatment.

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